Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference.

[0385] One preferred application supported by the data in TABLE 5 is the use of the ERγ for diagnosing and/or screening for breast cancer. Measurement of the ERγ specifically will provide a more accurate determination of estrogen receptor status and therefore permit more precise modeling of the therapy for each patient. ERγ will be identified by immunohistochemical methods, labeled ligand binding with very high specific activity isotopes, and by PCR and other molecular biology analyzes. Other methods will also be applied. Similar analyses are expected to be applicable to other estrogen receptor related or estrogen receptor containing mucosal cancers including ovarian, uterine, vaginal, cervical, colon, lung, stomach, pituitary, liver, pancreas, skin and kidney, as described in co-owned, concurrently filed U.S. Patent App. No. 09/852,547 (Atty. Dkt. No. 1944-00800)/PCT/US2001/015171 (Atty. Dkt. No. 1944-00801) entitled "Compositions and Methods for the Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference.

HD 2/2/11

[0386] Fc receptors are so named because they bind specific heavy chains (Fc domains). However, before coming to this conclusion, it should be emphasized strongly that the Fc family represented by Fcγ (IgG), Fcα (IgA), and Fcμ (IgM) have traditionally been considered to be located on lymphoid series cells (Fridman WH (1991) *FASEB J* **5**, 2684-2690; Raghavan M and Bjorkman PJ (1996) *Annu Rev Cell Dev Biol* **12**, 181-220). There is only limited experimental support for the concept that these receptors are located on epithelial cells (Tonder O *et al.* (1976) *Acta Pathol Microbiol Scand* **84**, 105-111). For the family of leukocyte IgG receptors, 12 transmembrane or soluble receptor isoforms are known. These are grouped into three classes FcγR1 (CD64), Fcγ RII (CD32) and Fcγ RIII (CD16) (Valerius T *et al.* (1997) *Blood* **90**, 4485-4492). For IgA, there is one gene that encodes several receptors) (*i.e.* Fcα) by alternate splicing to yield forms from M_r 55,000 to 110,000 (Pleass RJ *et al.* (1996) *Biochem J* **318**, 771-777; van Dijk TB *et al.* (1996) *Blood* **88**, 4229-4238; Morton HC *et al.* (1996) *Immunogenetics* **43**, 246-247). One of these, FcαR1 is constitutively expressed on monocytes and macrophages and other leukocytes. It binds IgA1 and IgA2 with about the same affinity. The receptor for IgM (*i.e.*Fcμ) is less well defined, but still has been partially characterized as a M_r 60,000 protein present on activated B cells and other B series cells (Ohno T *et al.* (1990) *J Exp Med* **172**, 1165-1175). The Fc superfamily has another very important aspect pertinent to this disclosure. Receptors of this family mediate negative effects on cells (Cambier JC (1997) *Proc Natl Acad Sci USA* **94**, 5993-5995). These receptors have an intracellular amino acid sequence motif I/VxYxxL (SEQ ID NO:1 and SEQ ID NO:2) described as an immunoreceptor tyrosine-based inhibitory motif (ITIM) that signals cell growth shutdown after ligand binding. These signals have

been characterized in the FCγRIIB1 receptors of human and mouse (Olcese L *et al.* (1996) *J Immunol* **156**, 4531-4534). The hallmark of these ITIM receptors is that they shut off growth factor dependent growth.

HD 2/2/11

[0041] The compositions, methods and models of the present invention overcome major shortcomings of the prior art and satisfy long-felt needs for, among other things, a sensitive way to screen substances for estrogenic and androgenic effects. It was discovered, and the embodiments herein demonstrate, that the immune system plays a major role in the growth of estrogen responsive breast and androgen responsive prostate cancers, as well as cancers of other steroid and thyroid hormone responsive mucosal epithelial tissues. IgA, IgM and certain IgGs provide negative regulation of steroid hormone responsive mucosal epithelial cancer cell growth, including breast, prostate, pituitary, kidney and other glandular cancer cells. For the purposes of this disclosure, "cell growth" means cell proliferation or an increase in the size of a population of cells through mitogenesis and cell division rather than an increase in cytoplasmic volume of an individual cell. Prior to the present disclosure, no growth regulating role was known for the secretory immune system, which produces predominantly immunoglobulin A (IgA) and immunoglobulin M (IgM) and lesser amounts of immunoglobulin G (IgG). The discovery that IgA and IgM are the major negative regulators of steroid hormone responsive cell growth arose out of the Inventor's work directed at purifying breast cancer regulatory factors from biological fluids, as described in the following Examples. This discovery and the present invention constitute a major breakthrough in the understanding of these cancers, and other glandular/mucosal tissues that secrete or are bathed by polymeric IgA, secretory IgA (sIgA), IgM and certain IgGs. For the first time, a direct link has been established between the secretory immune system (IgA and IgM) and the most prevalent types of cancer that occur throughout the world. Binding of IgA and IgM to the poly-immunoglobulin receptor (i.e. poly-Ig receptor or poly IgR) is an important step in carrying out the regulatory function of IgA and IgM, and initial indications are that poly IgR mediates the negative regulation of steroid hormone dependent cell growth. Application of these scientific breakthroughs to the detection, diagnosis, prognosis, treatment and deterrence or prevention of cancer of mucosal epithelial tissues (e.g., breast, prostate, kidney, pituitary, thyroid and colon) is described in U.S. patent application Ser. No. 09/852,547 (Atty. Dkt. No. 1944-00800) PCT/US2001/015171 (Atty. Dkt. No. 1944-00801) entitled "Compositions and Methods for the Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference.

HD 2/2/11

[0175] **Fig. 87.** Protein Sequencing Results with CA-PS-Pool II Peptides and Homology to Human SHBG, Rabbit SHBG and Rat and Hamster Androgen Binding Protein (SEQ ID NOS:3-19).

HD 2/2/11

[0184] **Fig. 96.** Protein Sequencing Results with Rat "SHBG-like" Peptides and Homology to Human SHBG, Rabbit SHBG and Rat and Hamster Androgen Binding Protein (SEQ ID NOS:36 and 20-26).

HD 2/2/11

[0237] To facilitate review of the detailed description of preferred embodiments, a Table of Contents is provided. The titles used for the various subsections and examples are not intended to be limiting and are only an aid to locating certain subject matter. In addition, each Example begins with a short summary of that Example, which is intended merely to facilitate review and is not limiting on the disclosure contained in the full Example, and ends with a Discussion of some conclusions that may be drawn from that Example.

Table of Contents

HD 2/2/11

230

[0~~240~~] It has been discovered that the negative regulators of steroid hormone responsive cancer cell growth (estrogen reversible inhibitors) in serum are products from the secretory immune system, i.e., the ~~immunologobulins~~ immunoglobulins A (IgA), M (IgM) and IgG1. These "immunoglobulin inhibitors" act as steroid hormone and thyroid hormone reversible inhibitors of mucosal cell growth. There has been no previous identification of secretory immune system immunoglobulins as regulators of epithelial (mucosal) cell growth, and this discovery is unique in the cell growth regulation field. Application of certain of the compositions and methods is expected to relate to 80% of all human cancers because this high incidence rate arises from mucosal tissues. There is no previously reported evidence directly linking the secretory immunoglobulins with regulation of mucosal cell growth.

HD 2/2/11

295

[0~~363~~] Using the present 34°C CDE and XAD-4 serum, estrogen responsiveness can be demonstrated in rat tumor cells, where no such responsiveness had previously been demonstrated. Further, because estrogen responsiveness and binding affinity can now be compared, data indicating the existence of [[an]] a heretofore unknown estrogen receptor have been generated.

HD 2/2/11

306

[0~~385~~] One preferred application supported by the data in TABLE 5 is the use of the ERγ for diagnosing and/or screening for breast cancer. Measurement of the ER.gamma. specifically will provide a more accurate determination of estrogen receptor status and therefore permit more precise modeling of the therapy for each patient. ERγ will be identified by immunohistochemical methods, labeled ligand binding with very high specific activity isotopes, and by PCR and other molecular biology analyzes. Other methods will also be applied. Similar analyses are expected to be applicable to other estrogen receptor related or estrogen receptor containing mucosal cancers including ovarian, uterine, vaginal, cervical, colon, lung, stomach, pituitary, liver, pancreas, skin and kidney, as described in co-owned, concurrently filed U.S. Patent App. No. 09/852,547 (Atty. Dkt. No. 1944-00800)/PCT/US2001/015171 (Atty. Dkt. No. 1944-00801) entitled "Compositions and Methods for the Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference.

HD 2/2/11 318 [0407] It is widely believed that the phenol red indicator in tissue culture medium acts [[is]] as a weak estrogen (Berthois Y *et al.* (1986) *Proc Natl Acad Sci USA* **83,** 2496-2500). In the first report describing the phenol red problem, the indicator itself was thought to act as an estrogen (Berthois Y *et al.* (1986) *Proc Natl Acad Sci USA* **83,** 2496-2500). At the concentration in standard culture media (*e.g.* D-MEM/F-12 is 8.1 mg/mL or 22.9 μM), it was believed to stimulate ER^+ cell growth nearly as well as natural estrogens. Simply stated, this meant that exogenous estrogens would have no effect because the cells were already nearly completely stimulated. Further work by the original investigators later demonstrated that it was the lipophilic impurities in phenol red that were the true culprits (Bindal RD *et al.* (1988) *J Steroid Biochem* **31**, 287-293). The chemical structure of one was determined to be bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane (Bindal RD and Katzenellenbogen JA (1988) *J Med Chem* **31**, 1978-1983). Interfering amounts of the impurities were identified in many different commercially available preparations of phenol red (Bindal RD *et al.* (1988) *J Steroid Biochem* **31**, 287-293; Bindal RD and Katzenellenbogen JA (1988) *J Med Chem* **31**, 1978-1983). These investigators concluded that many, if not most, phenol red containing culture media had sufficient contaminants to at least partially mask estrogenic effects. Despite the wide acceptance of phenol red as an estrogen, experience has shown differently. Instead, large estrogen mitogenic effects have been observed in phenol red containing culture medium with ER^+ MCF-7 human breast cancer cells, T47D human breast cancer cells, MTW9/PL2 rat mammary tumor cells, GH rat pituitary tumor cells, H301 Syrian hamster kidney tumor cells and the androgen receptor positive (AR^+) and ER^+ LNCaP human prostatic carcinoma cells, as shown herein and subsequently reported (Moreno-Cuevas JE and Sirbasku DA (2000) *In Vitro Cell Dev Biol* **36**, 410-427; Sirbasku DA and Moreno-Cuevas JE (2000) *In Vitro Cell Dev Biol* **36**, 428-446, incorporated by reference). In these studies, even when phenol red was present, estrogen-inducible cell number increases of 8 to 80-fold were observed. These responses were as large or larger than any previously reported. They exceeded any reported in phenol red free medium. Also, growth without the natural hormone was very limited even with phenol red present (Moreno-Cuevas JE and Sirbasku DA (2000) *In Vitro Cell Dev Biol* **36**, 410-427; Sirbasku DA and Moreno-Cuevas JE (2000) *In Vitro Cell Dev Biol* **36**, 428-446).

HD 2/2/11 460 [0667] **Effects of CA-PS-pool II on ER^+ Cell Growth in Serum-free Defined Medium.** The estrogen reversible inhibitory effects of CA-PS-pool II were examined with eight ER^+ cell lines growing in different serum-free defined media **(Fig. 86)**. The cell lines were the MCF-7K cells **(Fig. 86A)**, the T47D cells **(Fig. 86B)**, the ZR-75-1 human breast cancer cells (ATCC# CRL-1500) **(Fig. 86C)**, the GH_1 (ATCC# CCL-82) **(Fig. 86D)**, GH_3 (ATCC# CCL-82.1) **(Fig. 86E)**, and GH_4C_1 **(Fig. 86F)** rat pituitary tumor cells, the MTW9/PL2 rat mammary tumor cells **(Fig. 86G)**, and the H301 Syrian hamster kidney

tumor cells (**Fig. 86H**). At 20 to 30 μg/mL, this fraction completely inhibited growth. The inhibition was totally reversed by 10 nM E_2. The E_2 effects on cell number were in the range from 33 to 72-fold (*i.e.* CPD = $2^{5.04}$ to $2^{6.18}$). The activity was not replaced by serum albumin at 5 mg/mL (data not shown). The estrogen mitogenic effects seen in defined medium containing only a few μg/mL of protein were equal to or greater than those seen in medium containing 30 to 50% (v/v) CDE-horse serum with every ER^+ cell line tested (**TABLE 10**). Plainly, the serum-free conditions established herein are the most defined model assay systems yet established to demonstrate estrogen responsiveness *in vitro*.

HD 480
2/2/11

[0702] The identification of IgA and IgM as serum-borne inhibitors fully separates these inhibitors from the teachings of U.S. Pat. Nos. 4,859,585 (Sonnenschein) and 5,135,849 (Soto), which arrived at no molecular identification of the inhibitor. The series of investigations presented above demonstrate that a very longstanding problem has been solved. While the solution is significant, an even more an important consequence of this knowledge is the fact that for the very first time, mucosal cell hormone dependent growth has been linked to a natural immune regulation. Moreover, this information has direct application to the diagnosis, genetic screening, prevention and therapy of breast and prostate cancer and a high likelihood of applications to other mucosal cancers, as described in more detail in U.S. patent application Ser. No. 09/852,547 (Atty. Dkt. No. 1944-00800)/PCT/US2001/015171 (Atty. Dkt. No. 1944-00801) entitled "Compositions and Methods for the Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference.

HD 526
2/2/11

[0774] In this Example, it is shown that the inhibiting effects of IgG1κ were mediated by an Fc receptor or Fcγ-type receptor. It is highly unlikely that IgG1 acts via the poly-Ig receptor. The poly-Ig receptor has a requirement for "J" chain for binding (hence its specificity for dimeric/polymeric IgA or pentameric IgM each of which have one J chain). Also, as shown in **TABLE 12**, Fcγ receptors are localized in leukocyte series or bone marrow origin cells. There is no convincing evidence of their presence in epithelial cells or in secretory cells of the mucosa. It is now proposed that the receptor being sought is one analogous to the Fcγ in at least two significant properties. First, it binds monomeric IgG1 via the Fc domain of the immunoglobulin with some participation of the κ light chain. Second, that the receptor has inhibitory activity akin to a new family of Fc receptors. The amino acid sequence of the new IgG1κ receptor is expected to have an immunoreceptor tyrosine-based inhibitory motif (ITIM) (VxYxxL) (SEQ ID NO:1 and SEQ ID NO:2) common to the new family of inhibitory motif receptors (Cambier JC (1997) *Proc Natl Acad Sci USA* **94**, 5993-5995). Other amino acid sequences may serve this same function.

528
HD 2/2/11 [0777] The amino acid sequence of a new Fc family receptor may include immunoreceptor tyrosine-based inhibitory motif (ITIM) common to a new family of inhibitory motif receptors (Cambier J C (1997) Proc Natl Acad Sci USA 94, 5993-5995). The results obtained in the present studies support the involvement of Fc receptors of mucosal cells that include one of the known members of the family of ITIMs or other amino acid sequences that serve this same function. Ongoing work includes genetic mapping to a specific chromosome number and locus. The genomic DNA sequence of the new receptor (or existing receptor if already known), introns and exons, will be obtained. This receptor may be used for diagnostic and clinical proposes, and as a screen for genetic susceptibility to breast and prostate and other mucosal cancers, as described in more detail in U.S. patent application Ser. No. 09/852,547 (Atty. Dkt. No. 1944-00800)/PCT/US2001/015171 (Atty. Dkt. No. 1944-00801) entitled "Compositions and Methods for the Diagnosis, Treatment and Prevention of Steroid Hormone Responsive Cancers," which is hereby incorporated herein by reference. Identification of mutations and changes associated with progression from normal cells to autonomous cancer cells is also expected, and, along with detection of changes in regulation of expression and allelic imbalances in the receptor gene, will have very useful research, diagnostic and clinical applications.

530
HD 2/2/11 [0781] As discussed above, serum contains a great variety of mitogenic agents. On this point the present results in 50% (v/v) serum were especially relevant. This concentration of serum is a rich source of mitogens including insulin and the insulin-like growth factors. Nutrients and other serum components also have growth-promoting effects. Examples include diferric transferrin, unsaturated fatty acids bound to albumin, complex lipids and ethanolamine. Clearly, the inhibitor(s) also blocks their growth effects, which lends support to the conclusion that the mediating receptor for the serum-borne agent must have special properties. For instance, the immunoreceptor tyrosine-based inhibitory motif (ITIM) class of Fc receptors is of particular interest with respect to identifying the mediator(s) of immunoglobulin inhibition of cancer cell growth, because the hallmark of the ITIM receptors is that they have an intracellular amino acid sequence motif I/VxYxxL (SEQ ID NO:1 and SEQ ID NO:2) that signals cell growth shutdown after ligand binding, and therefore shuts off growth factor dependent growth. In the preceding Examples it is demonstrated that steroid hormones are selectively capable of reversing the effects of the serum inhibitor(s). Plainly, as predicted by the estrocolyone hypothesis, serum contains an inhibitor(s) that has a dominant role in the regulation of proliferation of steroid hormone target cells. The isolated IgA and IgM blocked growth factor dependent growth in serum-free defined medium. Because of its "master switch" character, the newly identified immunoglobulin inhibitors have many important and useful applications. Moreover, the results of the present investigations support the view that the inhibitor(s) will have biological implications extending well beyond estrogen and androgen target tissues.

HO 538
2/2/11

[0790] To identify the receptors mediating the androgen reversible inhibition of normal and/or AR^+ cells, PCR cloning methods will additionally be used to determine the cDNA sequences of the poly-Ig receptor and Fcγ receptors from normal, AR^+ and, if indicated, from AR^- cells. This method will provide clear answers to the question of the relationship of the human poly-Ig receptor and Fcγ receptors to immune system negative regulation. It is expected that the receptors will be found to be either identical to known sequences or altered in sequence to convert them to "inhibitory motif" receptors. Based on the known cDNA sequence of the poly-Ig receptor from HT-29 cells, PCR cloning technology will be applied to obtain a full-length clone from the LNCaP and T47D cells. Ongoing investigations are directed to comparing receptor sequences from normal prostate and breast cells to identify any changes. Based on the known sequence of the FcγRIIB1 receptor, these same studies will be repeated. The receptors identified by cloning will be examined for the immunoreceptor tyrosine-based inhibitory motif (ITIM) amino acid sequence I/VxYxxL (SEQ ID NO:1 and SEQ ID NO:2) or related sequences. Concomitantly, the cells will be examined by Western analysis for SHP-1 and SHP-2 phosphatase mediators of the inhibition of growth factor activity. These markers are not only associated with the inhibitory motif but also other inhibitory receptors. More specifically, an LNCaP and T47D full-length poly-Ig receptor clone will be prepared and compared to the reported sequence of the poly-Ig receptor. The same technology will be applied to the poly-Ig receptor from normal prostate cells, and, if indicated, from the AR^+ lines. Because these cell lines are expected to express the known poly-Ig receptor, or a related form, the PCR approach is applicable. The same approach will be used with the Fcγ like receptor. However, in this case, because these receptors are predominantly lymphoid origin, the form in epithelial cells may be substantially different. Standard cloning methods will be employed to obtain the complete cDNA sequence of the Fcγ like receptor from normal and LNCaP cells. Total RNA will be extracted and mRNA purified by oligo dT cellulose chromatography (also for Northern analysis). cDNA synthesis will be done with oligo dT primers and AMV reverse transcriptase followed by Rnase H to remove RNA. Second strand synthesis will be done with hexameric random primers and DNA pol. I. Treatment with T4 DNA pol, Rnase H and Rnase A creates blunt ends. EcoR1 methylation is followed by EcoR1 linkers and ligation into a cloning vector. (Stragene) vectors based on λgt10 (hybridization screening) and λgt11 (secretory component antibody screening). Both vectors will accept inserts larger than the receptor. The cDNA sequence of human poly-Ig receptor known is the genomic sequence. These will be used to prepare sequence specific primers for PCR. The primers will encompass the 5' and 3' non-coding sequences to ensure a complete cDNA. The PCR products will be subcloned using the TA kit from Invitrogen. The sequencing of PCR clones will be done by the dideoxy chain termination method (Lone Star Labs, Houston, TX). From these, determination of whether there have been significant alterations in

the receptor during the transition from normal to ER$^-$ and AR$^-$ cancer cells is expected. From sequence data, the ITIM amino acid sequences indicating an inhibitory motif receptor will be sought. It is important to note, however, that the absence of these sequences does not necessarily rule out an inhibitory function. The Western analyses for SHP-1 and SHP-2 will be valuable for indicating an inhibitory function even in the absence of ITIM or when the ITIM is in a modified form.

HD 2/2/11 544

[0801] The receptors mediating the immune response regulation must be at or very near the beginning of the onset of breast cancer. Using the tools developed in the present series of investigations, it is expected that inhibitory motif receptors for these immunoglobulins will be identified. It is now proposed that the mediating receptors are members of the Ig superfamily, which includes Fc receptors and a new class of Ig inhibitory motif receptors. This new class of receptors has emerging importance because of the increasing recognition of the role of negative regulation of cell growth. These receptors have both common and unique properties. They bind immunoglobulins via the Fc domains and hence can be classified as Fc receptors. One of these is, in fact, FcγRIIB that binds IgG1 **(TABLE 12)** and causes inhibition of antigen activation of B cells. There are many other examples (Cambier JC (1997) *Proc Natl Acad Sci USA* **94**, 5993-5995). Among these are more than 15 receptors now designated Signal-Regulatory Proteins (SIRPs). These all express a special inhibitor motif of six amino acids (I/VxYxxL) (SEQ ID NO:1 and SEQ ID NO:2) that is now referred to as the "immunoreceptor tyrosine-based inhibitory motif" or ITIM. One of the most marked characteristics of the ITIM containing SIRPs is that this motif recruits two phosphatases (SHP-1 and SHP-2) to result in the inhibition of all growth factor dependent proliferation. This is similar to what was observed with IgG1, IgA and IgM and ER$^+$ breast cancer cells and AR$^+$ prostate cancer cells serum-free defined medium. This work is expected to aid in the identification of the missing genes for sporadic breast cancers and a more complete understanding of the cascade of gene changes that lead to complete loss of immune control of breast cell growth.

HD 2/2/11 549

[0807] The proposed model for progression of mucosal cancers from normal cells to fully autonomous cancers is based on the experimental results presented and is unique. No previous recognition has been published of the roles of IgA, IgM and IgG1 in breast, prostate, or other mucosal cancers. Application of this model has diagnostic implications. Breast, prostate and other cancers can be examined for content of the receptors for IgA, IgM and IgG1 to determine stage of the cancer. This information can be compared to the determination of estrogen receptor and progesterone receptor status to aid in decisions regarding immunotherapy with immune modulators or the immunoglobulins or the use of combined anti-hormone and immune therapy modalities. Tumors that are negative for all of the immunoglobulin receptors are prime candidates for gene therapy to replace the receptors and thereby reestablish immune surveillance,